Articles

Molecular Structures of Human Factor Xa Complexed with Ketopiperazine Inhibitors: Preference for a Neutral Group in the S1 Pocket

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The structures of the noncovalent complex of human factor Xa (fXa) with four non-peptide inhibitors containing a central sulfonylpiperazinone scaffold have been determined to about 2.1 Å resolution. Highly potent fXa inhibitors containing both neutral groups such as chlorobenzothiophene or chlorothiophene and basic groups such as benzamidine were shown to interact in the S1 pocket through the neutral group whereas the S4 pocket is occupied by the basic moiety. The scaffold comprising the sulfonyl keto piperazine moiety might play a pivotal role in the orientation of substituents, since there is a strong hydrogen bond between Gly219 of fXa and the carbonyl oxygen of the piperazine. This unique "reverse" binding mode is heretofore unreported in fXa and shows that electrostatic interactions in the S1 subsite are not an absolute requirement to maintain high affinity. Selectivity against other serine proteases can be readily explained in light of these structural results. It has opened up new prospects for designing fXa inhibitors with increased oral bioavailability.

Introduction

Myocardial infarction, stroke, deep vein thrombosis, and pulmonary embolism represent major causes of mortality in the industrialized world. Therefore, the prevention of blood coagulation has become a major target for new therapeutic agents. Efforts to find new antithrombotics have been directed at specifically inhibiting a serine protease along the coagulation cascade.[1] Factor Xa, which is located at the juncture of the intrinsic and extrinsic arms of the cascade, has been the subject of intensive research that has culminated in the discovery of non-peptidic small-molecule inhibitors of fXa.[2] It is thought that inhibition of fXa may be more effective than direct inhibition of thrombin in interrupting the coagulation cascade[3] and that fXa inhibitors may display less bleeding risk than α-thrombin inhibitors.[4] Consequently, fXa has emerged as an attractive target enzyme for new antithrombotic agents.[5]

A common approach to the design of fXa inhibitors has been built upon the knowledge of the X-ray structure of unliganded fXa[6] and upon the effort and success in developing α-thrombin inhibitors.[7] The reports describing crystal structures of fXa/ligands[8] have shown that basic aromatic group containing compounds bind in a canonical fashion typical of synthetic protease inhibitors, which is characterized by polar interactions in the S1 subsite with some hydrophobic contacts and by hydrophobic interactions in the aryl S4 binding site. In previous work,[9] a series of crystal structures of inhibitors complexed with fXa were determined in this laboratory to provide a suitable platform for structure-aided drug design. The inhibitors contain either a *β*-amino ester or a sulfonamidopyrrolidinone group as a central template for the presentation of ligands to the S1 and S4 subsites. These compounds, which incorporate a benzamidine group or a surrogate of the basic group (azaindole, aminoisoquinoline) as a P1 fragment, appeared to interact with the S1 pocket in different ways. These structural data have underscored that the formation of salt bridge interaction in the S1 pocket is not an absolute prerequisite for high affinity. In this paper, we report on the structures of potent fXa inhibitors containing a sulfonyl keto piperazine scaffold. Since preliminary SARs were difficult to rationalize, we determined the crystal structures of some representatives of this series in complex with fXa. Somewhat unexpectedly, highly potent fXa inhibitors containing both neutral groups such as chlorobenzothiophene and basic groups such as benzamidine were shown to interact in the S1 pocket through the neutral group whereas the S4 pocket is occupied by the basic moiety. This unique "reverse" binding mode is heretofore unreported in fXa, and it has opened up new prospects for designing fXa inhibitors with high potency and increased oral bioavailability.

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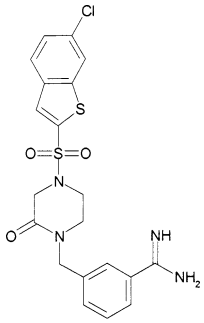
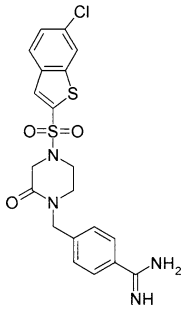
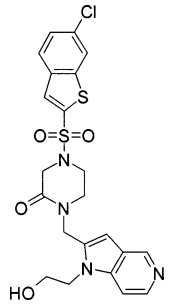
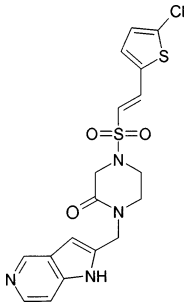
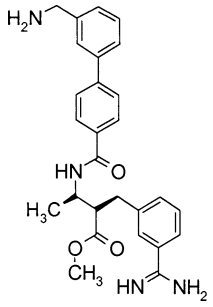
Table 1. Structures of the Factor Xa Inhibitors[20] **1** (RPR132747), **2** (RPR200095), **3** (RPR208944), **4** (RPR209685), and **5** (RPR128515)[a]

	1	**2**	**3**	**4**	**5**
structure					
K_i(fXa) (nM)	18	1.3	3.0	1.1	0.9
K_i(Tryp) (nM)	>2900	>2900	>2900	>2900	69
K_i(thrombin) (nM)	>4000	>4000	>4000	>4000	>3000

[a] The K_i for fXa, bovine trypsin, and human thrombin are given for each compound. They were determined as previously described.[21]

Binding Mode of Inhibitors to fXa (Table 1)

Compounds 1 and 2. Compounds **1** and **2** bind to fXa in an extended conformation with their chlorobenzothiophene group sitting in the S1 pocket and their benzamidine in the S4 pocket (Figure 1a). The structures of these compounds only differ in the position of the substitution on the aromatic ring of the benzamidine (*m*-benzamidine for **1** and *p*-benzamidine for **2**). Not surprisingly, they show strong similarities in their binding modes to fXa except for the benzamidine moiety, where some differences are found. The chlorobenzothiophene provides a snug fit in the S1 pocket and makes extensive van der Waals contacts with protein residues. The carbon−chlorine bond is directed toward the plane of the aromatic ring of Y228 (angle of 68°), and the chlorine atom lies at about 3.6 Å from the centroid of the ring, suggestive of some interactions. In a benzamidine-containing inhibitor/fXa structure,[9] a water molecule is observed at the position occupied by the chlorine atom, indicating that displacement of this solvent molecule is required for binding. The sulfonyl group makes van der Waals contacts with the aliphatic part of the Q192 side chain. The piperazinone linker is located above G216 and W215. Its carbonyl oxygen is involved in a hydrogen bond with the ñNH of G219. The benzamidine in both compounds fits in the S4 pocket with the aromatic ring perpendicular to the indole of W215 and parallel to the plane of F74. The aromatic ring of Y99 is almost perpendicular to that of the *p*-benzamidine of **2**, and there is an edge-to-face interaction between both rings as typified by an edge-to-centroid distance of 4.1 Å. In the complex with **1**, the Y99 aromatic ring has rotated by about 90° from the position observed with **2** and is almost parallel to that of the benzamidine at a distance of 5.5 Å, indicative of very loose stacking contact. The amidine nitrogens of **1** form a water-mediated contact with the carbonyl oxygen atom of E97. The amidine of **2** is engaged in a more complex network of weak interactions. One amidine nitrogen of **2** makes water-mediated contacts with the I175 and T98 carbonyl oxygen atoms and is also involved in a π-cation interaction with the indole of W215, as indicated by a distance of 3.2 Å to the centroid of the benzene ring. The other amidine nitrogen makes very weak H bonds with the protein: a direct H bond with the carbonyl of E97 (distance of 3.4 Å) and a water-mediated contact with the carboxylate of E97. The sum of these weak interactions observed for the *p*-benzamidine-containing compound could account for the 12-fold increase in inhibition potency when compared with the *m*-benzamidine compound.

Compound 3. The S1 pocket of fXa is occupied by the chlorobenzothiophene, and the hydroxyethylpyrrolopyridine lies in the S4 pocket. Not surprisingly, the interactions between **3** and fXa are very similar to those found for the identical atoms of **1** and **2** and only differ in the S4 pocket (Figure 1b). The pyrrolopyridine lies perpendicular to the W215 indole and is sandwiched by the aromatic residues F174 and Y99. There is a stacking interaction with F174 (4.0 Å) and an edge-to-face interaction with Y99 (distance from centroid to edge of 3.9 Å). The pyridine nitrogen makes a hydrogen bond, with the structural water molecule bridging I175 and T98. The hydroxyethyl is facing out toward the solvent, but the ethyl displays van der Waals contacts with F174. The hydroxyl group is involved in a water-mediated internal hydrogen bond with the piperazinone carbonyl oxygen atom.

Compound 4. Compound **4** binds with the chlorothiophene located in the S1 pocket and the pyrrolopyridine in the S4 pocket (Figure 1b). The chlorothiophene interacts with fXa in the same way as the chlorobenzothiophenes of **1**, **2**, and **3** with the chlorine atom sitting above Y228. The thiophene of **4** occupies the position of the benzyl group of the other inhibitors, and the ethene group sits in the same plane as the chlorothiophene, mimicking the conformation of the thiophene observed with **1**−**3**. The central sulfonyl keto piperazine scaffold of **4** makes similar interactions as observed in the complex with **1**−**3**. The pyrrolopyridine group fits in the S4 pocket as observed with **3** and does not induce any rearrangement of the side chains of protein residues. The pyridine nitrogen forms a hydrogen bond with the water molecule bridging I175 and T98 that is located at the entrance of the pocket. The pyrrolo nitrogen is facing out toward the solvent and does not interact with the protein.

Discussion

fXa interacts with the central sulfonyl keto piperazine template in a manner different from that with other scaffolds such as sulfonamidopyrrolidinone or β-amino

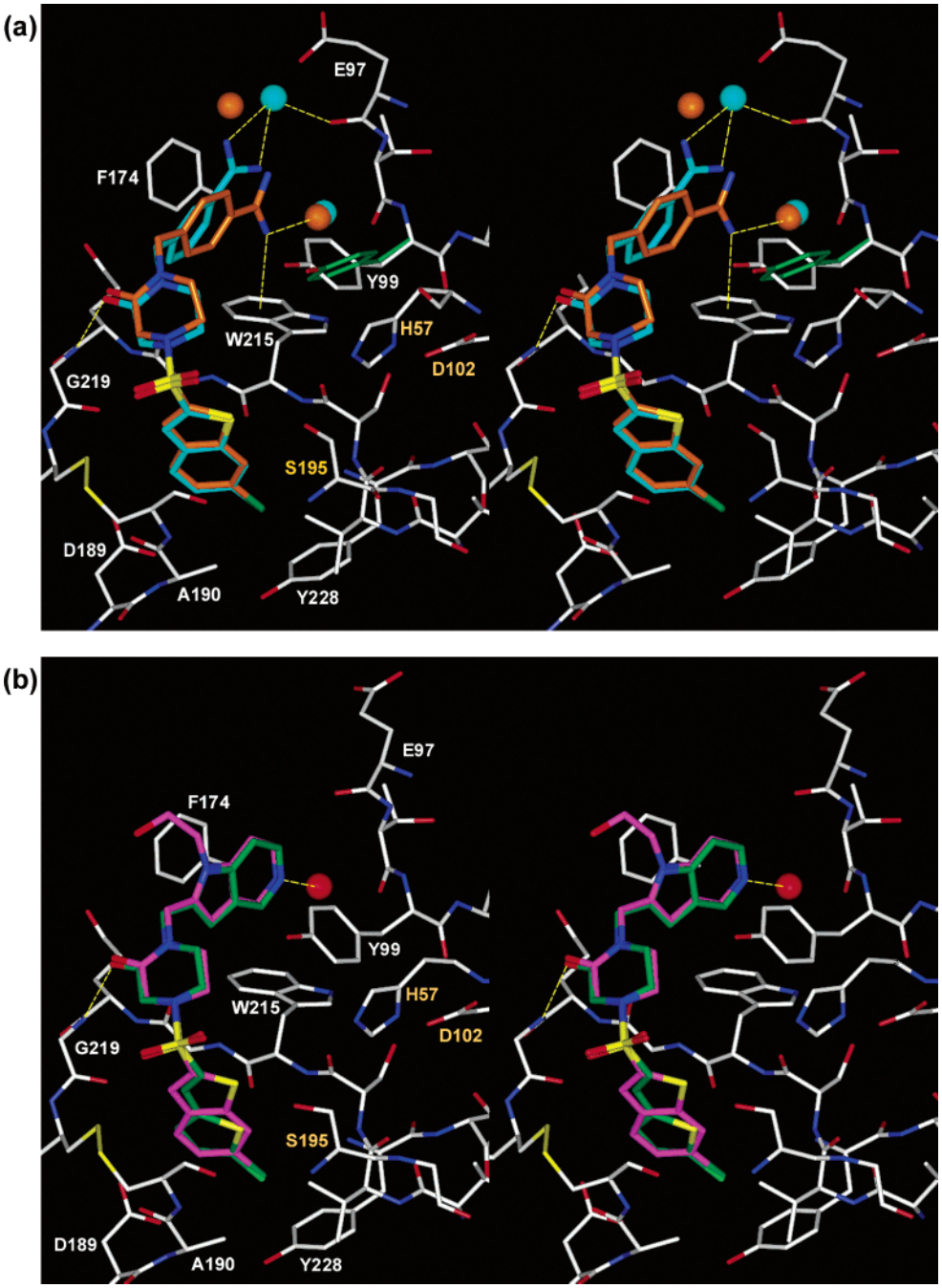


Figure 1. Binding interactions of inhibitors to fXa. The residues from fXa when bound with **1** defining the binding site are displayed with thin lines and with carbon, nitrogen, sulfur, and oxygen atoms displayed in white, blue, yellow, and red, respectively. H bonds between inhibitors and fXa are indicated by dotted lines. Labels are given for fXa residues. All inhibitors are represented with thick lines and with nitrogen, oxygen, sulfur, and chlorine atoms displayed in blue, red, yellow, and green, respectively. (a) Overlay of compound **1** (carbon atoms in light-blue) on **2** (carbon atoms in orange) when bound to fXa. Light-blue and orange balls represent water molecules as observed in the fXa/**1** and fXa/**2** complexes, respectively. Y99, which changes conformation upon binding to compound **2**, is displayed with carbon atoms in green. (b) Overlay of compound **3** (carbon atoms in magenta) on **4** (carbon atoms in green) when bound to fXa. Red balls represent water molecules as observed in the fXa/**3** complex.

ester.[9] The only conserved interaction found between these scaffolds and fXa is the H bond, which involves the nitrogen atom of G219 and the carbonyl oxygen of the keto piperazine moiety, suggesting that this contact might reflect one of the main anchoring points for fXa binding. It might also play a pivotal role in the orientation of substituents. The scaffold would appear to play the role of a linker that preserves the projection of the substituents into the S1 and S4 pockets, but its contribution to the binding energy is difficult to estimate.

The canonical binding mode of a synthetic trypsin-like serine protease inhibitor is characterized by two main interacting sites (S1 and S4): (1) formation of a salt bridge or H bonds between the basic group and D189 and hydrophobic contacts with the aromatic or aliphatic part of the inhibitor in the S1 pocket and (2) hydrophobic interactions in the aryl S4 binding site[1] (Figure 2a). This was found, for instance, for compound **5**[9] and for the Banyu inhibitors[8] when bound to fXa. This is also consistent with the fact that the natural

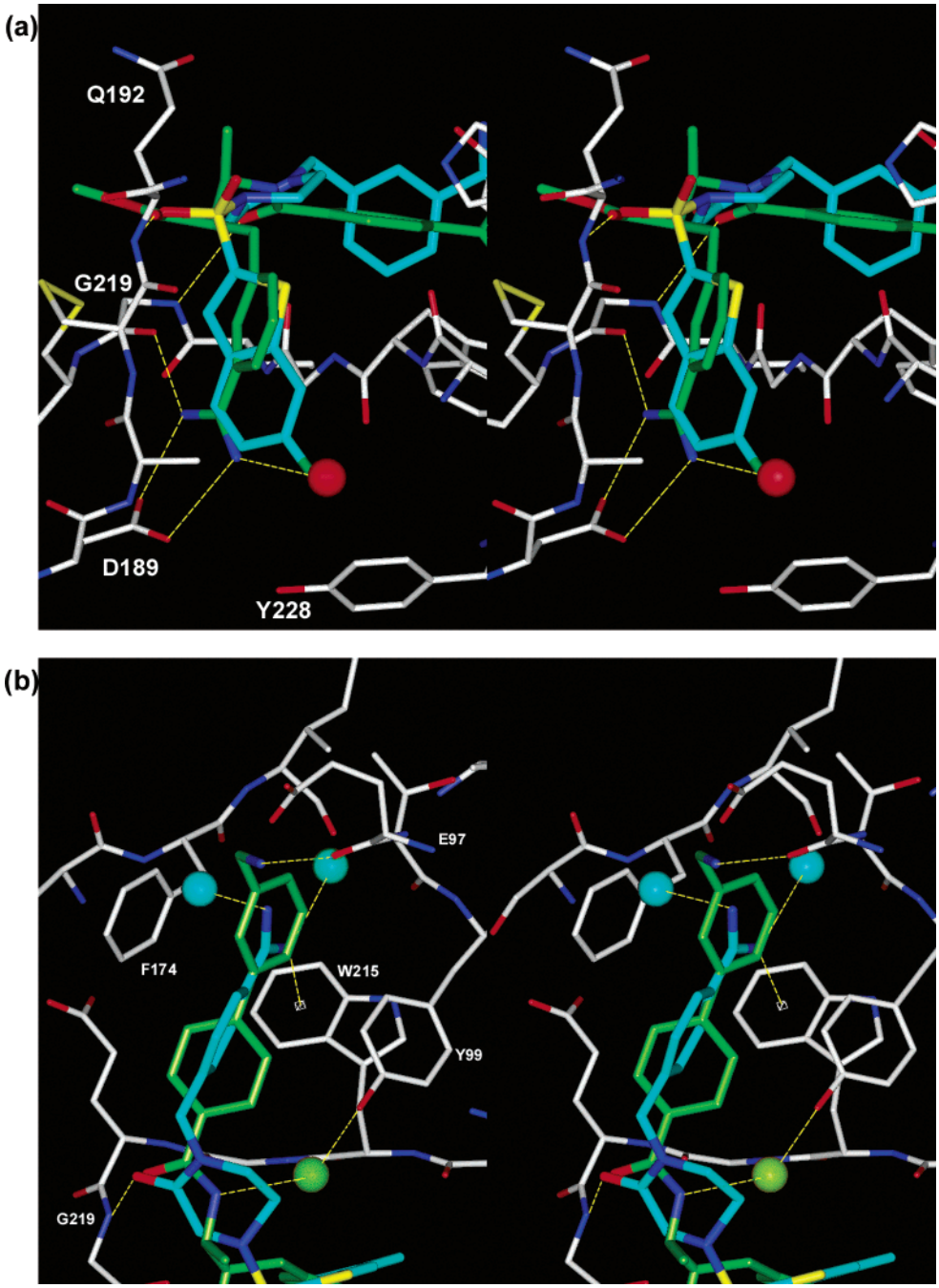


Figure 2. Comparison of the binding mode of **1** and **5** to fXa. (a) Close-up of the S1 pocket. Carbon atoms of **5** are exhibited in cyan. H bonds are indicated by yellow dotted lines for fXa complexed with **5**. Red balls represent water molecules as observed in the fXa/**5** complex. (b) Close-up of the S4 pocket. The π−cation interaction is indicated by orange dotted lines for fXa complexed with **1**. Green and light-blue balls represent water molecules as observed in the fXa/**1** and fXa/**5** complexes, respectively.

ligand comprises a highly basic arginine as a P1 group. Chlorobenzothiophene and chlorothiophene were attached to the central template with the hope that they would fit in the S4 aryl subsite. Molecular modeling docking studies indicate that **1** or **2** would be optimally positioned with the benzamidine moiety in the S1 pocket and the chlorobenzothiophene group in the S4 pocket. Crystallographic analysis has shown that this binding mode is not observed and that they interact with fXa in a "reversed" way. Standard force field methods are certainly challenged to model quantitatively both the cation−π interaction and the chlorine−aromatic interaction, and this aspect might explain why docking experiments have failed to predict the "reverse" binding mode as the top-scoring model. To determine if the chlorine−aromatic interaction exists because steric factors place the C−Cl bond in an appropriate position close to the aromatic ring, a search of the Cambridge Crystallographic Structural Database was performed to estimate the frequency and preferred orientation of such an interaction.[10] By use of a 4.5 Å distance cutoff between the centroid of the aromatic plane and a chlorine atom attached to an aromatic ring and a constraint on the angle between the C−Cl bond and the centroid of the ring (60°−120°), over 400 potential Cl−aromatic interactions were selected. The most common angle between the C−Cl bond and the aromatic ring is about 88°, and the distribution of the distance from Cl

Table 2. Crystal and Diffraction Data of Human Factor Xa with Inhibitors

	fXa/**1**	fXa/**2**	fXa/**3**	fXa/**4**
crystal parameters				
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a (Å)	55.0	56.0	56.5	55.9
b (Å)	71.8	71.7	72.3	71.7
c (Å)	79.0	79.0	79.7	79.0
resolution (Å)	2.05	2.1	2.05	2.1
R_{sym}[a] (%)	3.2 (11.8)	5.8 (28.4)	5.0 (13.5)	5.8 (14.7)
completeness[a] (%)	92.0 (89.4)	93.5 (96.2)	86.8 (67.3)	96.9 (98.4)
no. of reflections, redundancy	18808, 2.4	17692, 2.4	18225, 3.1	18587, 2.3
reflections > 5σ (%)	83.2 (73.2)	76.0 (56.1)	85.2 (76.1)	78 (48.7)
refinement				
no. of protein atoms (occupancy ≠ 0)	2130	2180	2171	2154
average *B* value for protein atoms and ligand atoms (Å^2)	29.0, 45.0	22.0, 22.0	24.5, 29.0	27.0, 29.0
range of data	20.0−2.05	25.0−2.1	30.0−2.15	20.0−2.1
R value	22.5	20.3	21.5	20.0
weighted rmsd from ideality				
bond length (Å)	0.007	0.006	0.007	0.006
bond angle (deg)	1.32	1.29	1.27	1.27

[a] Figures in parentheses represent statistics in the last shell of data (highest resolution).

to the centroid of the ring shows a preferred value of about 4.03 Å. These values are to be compared with 66° and 3.6 Å for the complex of **1** and fXa and would show that the observed binding geometry is relatively close to the most common orientation. Semiempirical quantum mechanics computation on compound **1** as implemented in MOPAC[11] indicated that the partial charge of the chlorine atom is close to nil. This would suggest that there is almost no electrostatic interaction between the aromatic ring of Y228 and the chlorine atom but that there are solely van der Waals contacts. In previous studies, it was shown that surrogates of the benzamidine (aminoisoquinoline, azaindole) could bind in the S1 pocket of fXa, but unlike the chlorobenzothiophene groups of **1**−**3**, they were not able to displace a buried water molecule located at the bottom of the S1 site. Taken together, this would indicate that the observed lipophilic interaction in fXa is likely to be favorable, although an accurate estimate of its contribution to the binding energy remains very challenging.

It was recently reported[12] that selectivity for the S1 site of Ser190 trypsin-like serine protease could be achieved through introduction of a chloro group ortho to the arylamidine of inhibitor scaffolds. This halo-substitution resulted in enhanced specificity between tissue type plasminogen activator (Ser190 protease) and urokinase (Ala190 protease) via displacement of the water molecule at the bottom of the S1 pocket. The gain in selectivity against Ala190-containing serine protease was attributed to the deficit in hydrogen bonding of the arylamidine inhibitor. However this effect would appear to depend on the nature of the aryl scaffold, since introduction of this halo group would affect both the pK_a of the amidine and the geometry of the interactions because the relative location of a particular inhibitor scaffold could vary by over 1.0 Å. The fact that enhancement of selectivity in the S1 pocket by introduction of a chlorine atom can be achieved via displacement of a firmly bound water molecule on Ala190 protease (fXa, this work) with neutral scaffold and on Ser190 protease (tissue type plasminogen activator[12]) with arylamidine scaffolds highlights that a simple modification of the P1 group can result in a potent and selective inhibitor despite highly similar S1 sites of trypsin-like serine proteases. In both cases the entropy change upon displacement of the water molecule is likely to be close to maximal (2.0 kcal/mol[13]).

Interestingly the basic group of the benzamidine is well tolerated in the S4 pocket, exhibiting a preference for a substitution in the para position for the amidine. In this position, the amidine group can be engaged in a π−cation interaction with the indole of W215 (Figure 2b). It has been recognized that the aromatic amino acids Phe, Tyr, and Trp in particular are certainly hydrophobic but also highly polar (quadrupolar); thus, they can be involved in favorable interactions with a positive charge.[14] A recent survey in the protein data bank indicates that one-fourth of all tryptophan residues experience an energetically significant cation−π interaction. It was found that the S4 subsite of fXa did not exhibit hydrophobic character.[15] The carbonyl oxygen atoms of K96 and E97 with the side chain of E97 form a "nitrogen cation hole" at the periphery of the S4 site that has been invoked to explain the potency of inhibitors with positively charged and/or basic moieties at the P4 position.[8] Although the benzamidine moiety of **2** makes some use of these H-bond acceptors, the major electrostatic anchoring point in the S4 site would appear to be the π-electrons of the indole of W215. This would support the fact that the S4 pocket can favorably accommodate a positively charged aromatic group.

High specificity of the chlorobenzothiophene-containing compounds against trypsin can be readily explained in terms of the structural results. Trypsin exhibits a smaller S1 site because A190 from fXa is substituted for S190 and consequently cannot accommodate such a bulky group without adverse steric intereactions.

Conclusion

In summary, we have determined the crystal structure of fXa in complex with potent and selective inhibitors. The compounds show a "reverse" binding mode when compared with the previously described structures that rely on electrostatic interactions in the S1 pocket and aromatic contacts in the S4 site. Interestingly, it has already been reported that some noncovalent thrombin inhibitors could exhibit high potency while utilizing nonbasic groups in the P1 position.[16] Taken together, this could challenge the conventional thinking that the key interaction in the S1 subsite is principally driven

by the electrostatics. It also raises the question of the general applicability of such a finding to other types of serine proteases.

It is known that arylamidine-containing molecules often display poor oral absorption, and one of the main promising strategies followed up in the fXa and thrombin areas consists of reducing the basicity of the molecules.[5] The fact that inhibitors containing neutral groups could be accommodated in the S1 pocket while retaining significant potency should enable the design of molecules with increased oral bioavailability.

Experimental Section

Preparation of the Crystals. Purified human fXa was prepared as previously described.[9] The synthesis of compounds **1**−**4** is described by Choi-Sledeski et al.[9a] and that of compound **5** has been previously reported.[9b] For crystallization experiments, the protein was concentrated to 8 mg/mL in 5 mM Mes-NaOH, pH 6.0, 5 mM $CaCl_2$, and 1 μM compound **5** on a Vivaspin ultracentrifugation unit (Vivascience, France). Drops were prepared by mixing 3 μL of protein solution and 3 μL of a reservoir solution containing 18−20% PEG 600 and 50 mM Mes-NaOH, pH 5.7. Crystals were grown using the hanging drop/vapor diffusion method at 19 °C, and they appeared in a few days. Seeding cycles were necessary to improve the size and shape of the crystals.

X-ray Data Collection, Processing, and Crystallographic Refinement. Crystals were soaked in 50 μL of the mother liquor containing 1−1.5 mM inhibitor and 5−10% dimethylformamide (DMF) for 24h−72 h to displace **5** present during the crystallization. For data collection at 95 K, the crystals were gradually transferred into a 50 μL drop composed of the mother liquor supplemented with 1.0−1.5 mM inhibitor, 5−10% DMF, and 10% glycerol. Crystals were picked up with a fiber loop and flash-cooled in a stream of gaseous nitrogen at 95 K. The X-ray intensity data were collected on DIP2000 imaging plate (Mac Sciences, Japan) mounted on a FR591 rotating anode (Nonius, The Netherlands) operated at 50 kV and 90 mA. Data processing and scaling were carried out using MOSFLM[17] and SCALA.[18] Crystal data are presented in Table 2.

The structures were solved by molecular replacement. The search model was made from the coordinates of the refined structure of liganded fXa[9] with the inhibitor and solvent molecules removed. Energy-restrained least-squares refinement was carried out using X-PLOR.[19] Solvent molecules were included if they were on sites of difference electron density with values above 2.5σ and if they were within 3.5 Å of the protein molecule or of a water molecule. All atoms of the inhibitors and of fXa lie in well-defined electron density except the EGF1 domain that was not visible. The statistics of the crystallographic refinement are shown in Table 2. Atomic coordinates have been deposited with the Protein Data Bank (1NFU, 1NFY, 1NFX, 1NFW).

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